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Evaluation of Amoxicillin and Sulfonamide removal by *Bacillus cereus*, *Enterobacter ludwigii and Enterobacter* sp.

G. Yasodara Liyanage[1] *and Pathmalal M. Manage*[1*]

[1]*Department of Zoology ,University of Sri Jayewardenepura ,Sri Lanka*

Abstract

Most antibiotics are prone to release to the environment due to improper usage. The present study reports the biodegradation Amoxicillin (AMX) and Sulfonamide (SDI) by *Bacillus cereus*, *Enterobacter ludwigii* and *Enterobacter* sp. strains. These trains were previously reported to be degraders of crude oil. Different concentrations of AMX and SDI (0 - 420 ppm) were used to detect Minimum Inhibition Concentration (MIC) by 96 well plate method. Removal of the antibiotic was studied by introducing 0.5ml of starved bacterial suspensions into sterilized freshwater containing each antibiotic at 60ppm and 120ppm accordingly and incubated at 28^{0}C with shaking at 100rpm. 0.5ml sample aliquots were removed at 2 days interval for 14 days and analyzed by HPLC. The MIC values for SDI and AMX were recorded as 240ppm, 420 ppm for *B. cereus*, 120 ppm, 360 ppm for *E. ludwigii* and 180 ppm and 300ppm for *Enterobacter* sp., respectively. *B. cereus* strain completely removed AMX and 80% of SDI after 14 days of incubation.*E. ludwigii* showed 75% degradation for AMX and 60% for SDI, whilst, *Enterobacter* sp. degraded 80% (AMX) and 70% (SDI) respectively. Therefore these bacterial strains could be used as a useful bioremediation tool in the removal of antibiotics, contamination in the environment.

Keywords: Biodegradation /Amoxicilline /Sulfonamide/*Bacillus cereus/ Enterobacter ludwigii /Enterobacer* sp.

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1.Introduction

Over 70 million pounds of antibiotics are produced annually in the world, with approximately 60% for human use and 40% for animal husbandry (Sarmah et al., 2006). The excretion of incompletely metabolized antibiotics by humans and animals is the primary source of antibiotic in the environment. Other sources include waste released from hospitals, veterinary centers, industries producing antibiotics, sewage waste from households, animal feeds and agricultural farms where antibiotics are used to increase plant biomass (Rajilić-Stojanović et al., 2013).

The introduction of these compounds into the environmental through anthropogenic sources can constitute a potential risk for aquatic and terrestrial organisms. Common antibiotic contamination found in environment are ciprofloxacin,streptomycin, tetracycline , amoxicillin, sulphonamide, erythromycin, clarithromycin, sulfamethazine, augmentin and many others(De la Torre et al., 2012). Concentration of various antibiotics in different components of environment is highly variable. The use of antibiotics may also accelerate the development of antibiotic resistance genes (ARGs) and bacteria, which can lead to serious health risks to humans and animals (Kummerer, 2009). However, it was only inmid-1990s, when the use of these compounds was widespread and new analytical technologies were developed to quantify the amount of antibiotics (Lissemore et al., 2006).

Sulfadiazine (SDI) and Sulfamethoxazol (SMX) are sulfonamides which are a group of antibacterial agents commonly used in urinary tract infections, pneumocystis pneumonia, chronic branches and etc (BNF, 2010). For more than 80 years amoxicillin (AMX) and ampicillin (AMP), which belong to the penicillin group, has been widely used as an antimicrobial drugs for human therapy and are considered as the most important group of antibiotics. As a result of poor removal of amoxicillin in the drinking water and waste water systems, aquatic ecosystems are incurring changes as well as the development of antibiotic resistant bacteria and failure of treatment with antibiotic (Kummerer, 2009).

Most conventional waste water treatment processes are not designed for treatment of highly polar contaminants such as detergents and pharmaceuticals (Xu et al., 2007).Therefore, practical and economical solutions must be achieved in order to reduce the daily amounts of antibiotics discharged into the environment. Several methods such as absorption, incineration, oxidation-reduction, photolysis, hydrolysis, reverse osmosis and chemical degradation are possible for removing antibiotics from waste water in the environment (Jury et al., 2011). However these techniques are expensive and inaccessible to all parts of the world, especially to developing countries.

Bioremediation is an economically viable technology which may lead to degradation of antibiotics and produce simple compounds such as

*Corresponding author:
E-mail: pathmalalmanage@gmail.com

carbon dioxide, water nitrogen and organic materials. Therefore, many scientists have reported promising environmental friendly antibiotic degradation methods using native aquatic bacteria. Huys et al., (2000) reported that the tolerance of *Acinetobacter*, *Stenetrophomonas maltophilia* and *Aeromonas veronii* to AMX in fresh water environments of Ireland and England. Maki et al., (2006) has isolated Flavobacterium strains responsible for the degradation of a group of antibiotics including AMX and SDI. Moreover, Grundtet al., (2012) have reported AMX resistant activity by bacterial strain *Escherichia coli* as well. Interestingly, Kinbowale et al.,(2006) further reported the occurrence of SDI resistant bacteria isolates in mariculture environments.

Thus, the present study evaluates the AMX and SUF removal feasibility of *Bacillus cereus* (KM504128), *Enterobacter* sp. (KM4055978) and *Enterobacter ludwigii* (KM504129) strains, which were previously reported to be degraders of crude oil (Liyanage and Manage, 2015). If these bacterial genomes harbor multiple degradation genes which are responsible for different type of pollutants such as hydrocarbon and antibiotics, they can be used as a bioremediation tool.

2. Materials and methodology

2.1 Chemicals and reagents

AMX and SUF standards, HPLC and Bacteriological grade chemicals were purchased fromSigma Aldrich, USA.

2.2Determination of Minimum Inhibition Concentration (MIC)

The 5ml of LB broth culture was prepared for each strain by inoculating a loop of isolated bacteria and incubated overnight at 28^0C with continuous shaking at 100 rpm. The optimal density of the bacterial suspensions was equalized using 0.5 McFarland.

The 96 well plates were used to determine the MIC of antibiotic for each isolate. A well in the plate contained optimal density adjusted bacterial suspension (10 µl), LB broth and antibiotic (60ppm-480ppm). Each bacterial strain tested against AMX and SDI in triplicates. The 96 well plate was incubated while shaken at 28^0C and absorbance was measured at 0, 6, 12, 18 and 24 hours intervals by using an ELISA plate reader (Thermo Scientific, USA) at 590nm (Mulaudzi et al.,2011).The positive control wells in the plate received bacterial isolates and LB brothwhereas negative control received particular antibiotic, LB medium and sterilize saline solutions.

2.3 Determination of antibiotic degradation

B.cereus, *Enterobacter* sp. and *E. ludwigii* were transferred into 5 ml of liquid LB medium and incubated at 28^0C. The exponentially growing cultures were centrifuged at 1000 x g for 15 minutes. Bacterial suspension was re-suspended in 0.01M phosphate buffer saline (PBS)solution and incubated (28^0C, 100 rpm) overnight to deplete residual extracellular carbon content. Then samples were centrifuged at 1000 x g for 15minutes and the bacterial suspensions were washed three times using 0.01M PBS. Turbidity of all bacterial suspensions were equalized (A_{590}=0.35) using spectrophotometer (SPECTRO UV-VIS double beam PC).

A 0.5 µl of equalized bacterial suspension was inoculated into filter-sterile freshwater, containing AMX and SDI at a final concentration of 60ppm and 120ppm respectively. All flasks were incubated at 28°C with continuously shaking at 100 rpm. One milliliter of sub samples were collected at two days intervals for a period of 14 days. Then subsamples were centrifuged (12000 rpm) and supernatant of each sample was subjected to the frozen (-20°C) immediately. Then frozen samples were freeze-dried. Freeze dried samples were reconstituted in 1 ml of 100% aqueous HPLC grade Acetonitrile and subjected to the HPLC analysis. Control samples were prepared in triplicates without bacterial inoculation (Liyanage and Manage., 2014).

2.4 Analysis of antibiotics by HPLC

Analysis of antibiotics was carried out using the HPLC system consisting of Agilent 1200 series following the modified method of Fernandez-Torres et al. (2010). The injected volume was 20µl and chromatography was performed at 30^0C.The mobile phase considered of a mixture of 0.1% Glacial acetic acid in water (Component A): 0.1% Glacial acetic acid in acetonitrile (Component B), 99:1 (v/v) was pumped in beginning at a flow rate of 0.7 ml/min. Then followed linear elution gradient from 99% to 70% A in 25 min. Concentrations of each antibiotic were determined by calibration of the peak areas in UV detection range (300nm for SDI, 230nm for AMP) with an external standard. The HPLC method has a detection limit of 0.5µg/ml. AMX and SDI recoveries were greater than 90% with a relative precision of 15%.

3. Results

Antibiotic contaminated wastewater is not easily treated in biological wastewater treatment plants. One reason is that some antibiotics are not easily degradable in a normal treatment system and inhibit the many biological organisms in the treatment system. Specifically, tetracycline, sulfathiazole, and amoxicillin are representative antibiotics used in both human and veterinary medicine. As a solution microbial processes could be used for the remediation of explosives-contaminated soils and waste waters because it has been shown that a variety of different microorganisms are able to metabolize these chemical compounds.

AMX and SDI quantification was validated by the determination of the linearity of the calibration plot. The regression value for calibration plot of AMX was 0.990 while for SDI it was 0.992.

$C_{AMX} = (A_{AMX} - 16.3)/ 0.257$ → Equation 1
$C_{SDI} = (A_{SDI} - 1.234)/ 0.32$ → Equation 2

Equation 1 and 2 were derived from calibration plots for AMX and SDI and they were usedincalculation of relevant antibiotic concentrations in the degradation experiment. The peak area of the sample, (eg: - AMX = A_{AMX}), and the slope and intercept of each calibration curve was used to calculate the concentration of each antibiotic in unknown samples (eg:- AMX =C_{AMX}).

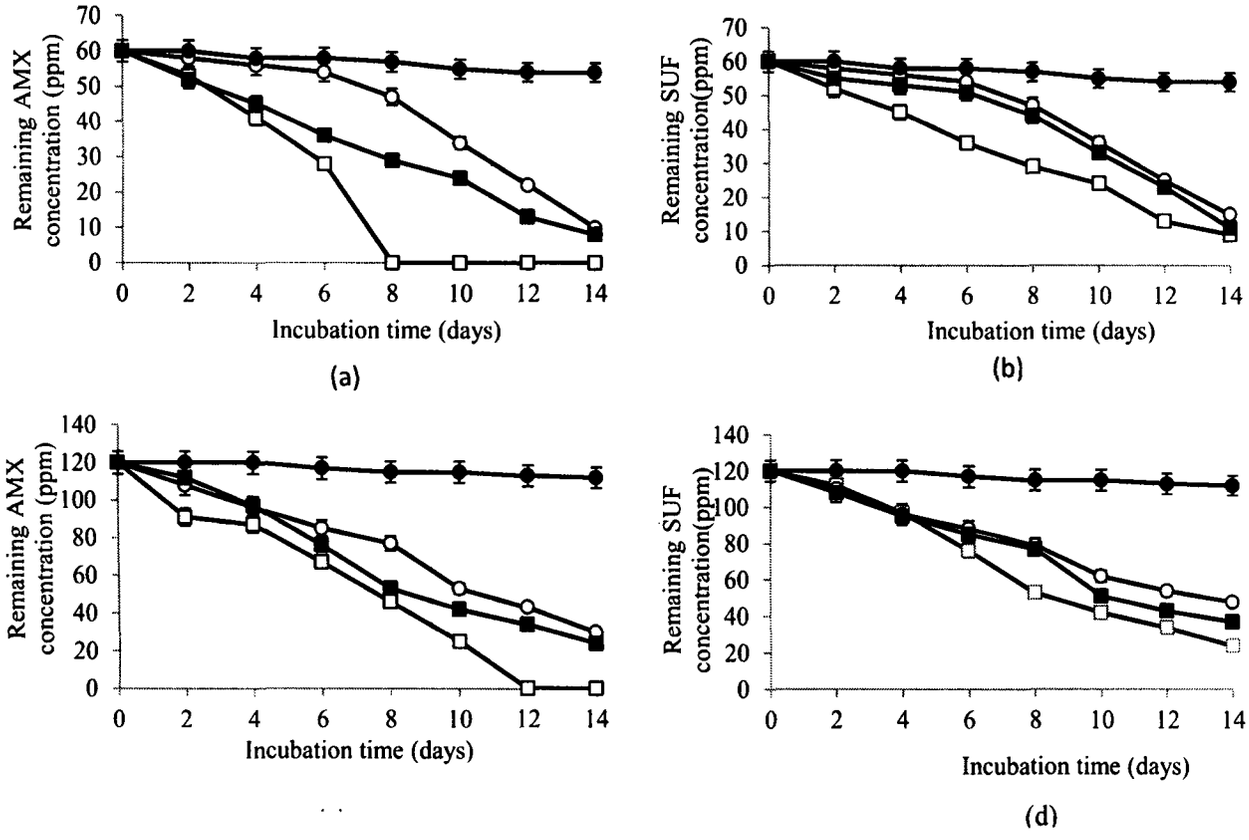

Figure1: Degradation of antibiotics at different concentrations (a) AMX degradation (60ppm); (b) SDI degradation (60ppm); (c) AMX degradation (120 ppm); (d) SDI degradation (120ppm). *Enterobacter* sp. (Closed square), *B.cereus* (Close Square), *E. ludwigii* (open circle) and control (close circle). When error bars are not shown, standard deviation was less than the width of symbol.

Figure 1 show the degradation of AMX and SDI by *B. cereus, E. ludwigii* and *Enterobacter* sp. during a 14 days incubation period at different antibiotic concentrations (60ppm, 120ppm).

When initial concentration of AMX was 60 ppm, *B. cereus* required 8 days for complete degradation of AMX, while *Enterobacter* sp. and *E .ludwigii* degraded 86.67% and 83.33% at 14 days of incubation respectively (fig 2 a ,c). Similar degradation pattern was followed by the bacterium isolates for AMX as well. Complete removal of AMX (120ppm) was detected within 12 days of incubation by *B. cereus* where *Enterobacter* sp. and *E. ludwigii* degraded 80% and 75%, respectively.Unlike AMX, SDI was unable to degrade completely because of the complex cyclic structure of SDI and this has resulted in retarded degradation rates for SDI. Therefore, complete degradation of SDI was not evident for all bacterial strains. At 60ppm of SDI, *B. cereus* showed highest degradation percentage (85%) where *Enterobacter* sp. (81.67%) and *E. ludwigii*(75%) showed descending trends (fig 2b). When initial concentration was 120ppm, *B. cereus*, *Enterobacter* sp. and *E.ludwigii* showed 80%, 60% and 53% reduction of SDI at 14 days of incubation respectively. Analysis of the sterile controls showed no significant loss of AMX or SDI (AMX- ~ 5%; SDI- ~ 7 %), slight decrease due to the environmental conditions was detected (Figure 1). These results confirmed that observed degradation was due to bacteria remediation.

At present study, antibiotics were not metabolized or modified in lag phases during degradation of AMX and SDI. Comparison of

AMX and SDI removal patterns of all three bacterial strains suggest that *B. cereus* shows a rapid removal rate with a steep slope (Figure1).

Furthermore, MIC was analyzed to see the effective dose of inhibition by all bacterial strains.The MIC values recorded for each bacterium are given in Table 1.

Table 1: MIC concentrations of AMX and SDI

Name of isolate	*MIC (ppm)*	
	AMX	SDI
B. cereus	420	240
E. ludwigii	360	120
***Enterobacter* sp.**	300	180

4. Discussion

Antibiotics are of concern due to potential genotoxic effects, disruption of aquatic ecology, promotion of antibiotic resistance, complication surrounding development of water reuse technologies, and possibly even increased human health risks (Jiang et al., 2013). Antibiotics in the environment at high concentration levels can lead to the development of antibiotic resistance bacteria. Some antibiotic resistance bacterial species found in antibiotic contaminated environment have evolved the ability to degrade different types of antibiotic in use of human and veterinary therapy (Sarmah et al., 2006).All findings obtained from the present study clearly demonstrated that microorganisms played a key role in the removal pathway of antibiotics. Initial concentration of 60ppm for AMX was completely removed by *B. cereus* (8 days), whereas *Enterobacter* sp. and *E. ludwigii* removed 86.67% and 83.33% respectively at 14 days of incubation. Each SDI isolate degraded more than 75%.

Bhusnurmath, (2010) reported, that antibiotics in penicillin group (AMX, AMP) can be degraded by β lactamase- producing bacteria like *Enterobacter* sp. Al-Wasify and Hamed (2014) reported that, after 30 days of incubation with *Corynebacterium* sp., *Flavobacterium* sp., *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Acinetobacter lwoffi* degraded 43%,41%, 77.8%, 76.7% and 74.3% of the AMX when initial concentration was 60ppm. Subathra *et al.*, (2013) reported that after 20 days of incubation *Bacillus* sp., (27.94%), *Micrococcus* sp., (27.69%), *Pseudomonas* sp. (39.68%), *Vibrio* sp. (3.64%) and *Achromobacter* sp. (3.24%) degraded AMX when initial concentration was 50ppm.

Costa et al., (2006) reported the SDI resistance activity and degradation shown by *Enterococuus* sp. isolated in sludge from municipal waste water treatment plant. Moreover, Grundt et al.,(2012), have reported AMX and SDI resistant activity by bacterial strain *Escherichia coli* as well.

Dallas et al., (2015) reported that AMX resistant by the bacterium *Acinetobacter, Alcaligenes, Citrobacter, Enterobacter, Pseudomonas, Serratia, Klebsiella* and *Proteus* showed MIC was greater than 256ppm. More or less similarly the present study records that MIC values for all three bacteria strains have greater than 300 ppm for AMX whereas greater than 180ppm for SDI respectively. Furthermore, Dallas et al., (2015) reported that *Bacillus* strain did not degrade AMX, but degraded some other antibiotics such as Cefotaxime and Sulfanomides. Interestingly, the results of present study show AMX degrading feasibility of *B. cereus*.

The antibiotics production was increased with the accelerated development of novel methods and technologies and that enhanced antibiotic contamination levels in environment.It is important to remark that treatment of water and residues from human activity is difficult to complete. Antibiotic-polluted residues are released in the environment without further processing at high levels. Thus monitoring of antibiotics in the environment and development of remediation processes is needed to minimize and prevent health issues caused by drug resistance bacteria in the environment.

5. Conclusion

Conventional wastewater treatment technologies are unable to achieve complete antibiotic removal for a broad spectrum of antibiotics, such as AMX and SDI. Therefore, microbial technologies can be used as an eco-friendly green solution for antibiotic pollution in environment. Among three isolates, the most dominant AMX and SDI degrading bacteria were identified as *B. cereus* and *Enterobacter* sp. respectively. Therefore, the results of this study showed that the bacteria are potential to remove the antibiotics from the environment.

Further researches studies related to antibiotics degradation can result in more efficient and less time consuming microbial technologies that are important for developing countries like Sri Lanka.

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